CARCINOGENICITY TESTING: PAST, PRESENT, AND FUTURE

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INTRODUCTION

This chapter presents an historical overview of cancer, carcinogenicity testing, and human cancer causes. Cancer has been known for a very long time, but the awareness of human carcinogenicity caused by chemicals is a phenomenon of the 20th century. This in turn has produced legislation that prohibits the use of carcinogens in the food chain and has provided guidelines for carcinogenicity testing in animals. Lifetime studies (18–24 months) in two main rodent species (rat and mouse), also known as the "Standard Chronic Bioassay," have been conducted since the 1960s.

Meanwhile, various deficiencies have been detected in the Chronic Bioassay; over-sensitivity is the major one. Hundreds of compounds have been tested with the Chronic Bioassay method, and about 50% have yielded (false) positive results. Lack of relevance to man has often been demonstrated by additional mechanistic studies. Additionally, more mechanistic and molecular knowledge has been gained in regards to the human carcinogenicity concept, including genotoxic versus epigenetic carcinogens, the multi-stage cancer theory, and human life style factors involved in carcinogenesis.

The above evolutions have opened up new opportunities for carcinogenicity testing, including short-term alternative carcinogenicity models. In addition, carcinogenicity testing is evolving from a standard chronic bioassay to a weight-of-evidence approach, where the mechanisms involved in rodent and human carcinogenesis are considered, and where communication between industry and regulatory authorities is encouraged.

CARCINOGENICITY TESTING IN THE PAST

History of Cancer, Carcinogenicity Testing, and Human Causes of Cancer

Cancer has probably existed as long as multicellular organisms have. Paleontologists have shown the presence of tumors in the bones of dinosaurs, long before the advent of *Homo sapiens*. The Ancient Egyptians knew of the existence of cancer in man. A papyrus with a hieroglyph that shows a clinical tumor was discovered. Autopsies of mummies have shown bone tumors in Egyptians. The first real descriptions of various tumors were those presented by Hippocrates in the 4th century B.C. He used the term "*carcinoma*," by which he meant a tumor that spreads and kills the patient. In the 1st century B.C., Galen made the distinction between "*natural tumors*," such as those present during the development of breasts in female adolescence and "*tumors, which go beyond the bounds of nature*," such as those related to bone healing following a fracture. Finally, he described "*unnatural tumors*," which today can be defined as a "neoplastic" growth of tissue. The views of Hippocrates and Galen dominated medicine for many centuries. In the 19th century, cancer knowledge expanded enormously, primarily through intensified studies on anatomy and histology. Bichat, and later Müller, confirmed Galen's theory. They postulated the hypothesis that cancer arises from accidental tissue formation. This in turn prompted Pasteur to propose his theory of "*omnis cellula et cellula*:" Every cell originates from another cell.

The discovery that exposure to exogenous chemicals could lead to cancer in humans was first made in the late 18th century, when Percival Pott demonstrated the relationship between cancer of the scrotum and the occupation of chimney sweepers exposed to coal tar/soot. Other examples noted later were scrotal cancers in cotton spinners exposed to unrefined mineral oils, and cancers of the urinary bladder in men who worked in textile dye and rubber industries due to their exposure to certain aromatic amines used as antioxidants. Experimental induction of cancer by chemicals was first reported in detail by Yamagiwa and Ichikawa in 1918, when repeated application of coal tar to the ear of rabbits resulted in skin carcinomas. Over the next few years, Kennaway and Leitch confirmed this finding and demonstrated similar effects in mice and rabbits from the application of soot extracts, other types of tar (e.g., acetylene or isoprene), and some heated mineral oils. These researchers also observed skin "irritation" sometimes accompanied by ulcers at the site of application of the test material. "Irritation" was thought to be an important factor in skin tumor development. However, not all irritants (e.g., acridine) induced skin cancer in mice and conversely, some purified

chemicals isolated from these crude materials produced a high incidence of skin tumors with little or no irritation. These observations suggested that intracellular changes induced by carcinogens might lead to cancer, independent of any lesions observable visually or by light microscopy. As a result, the hypothesis that cancer stems from the interaction of a chemical with a "sensitive" site in the cell was proposed. Further reading on the early history and concepts in cancer is provided by Van Cauteren et al. (1), Roe (2), and Grasso et al. (3).

By the mid 20th century, causes of human cancer related to exposure of chemical substances was of growing concern. Until the 1950s, the known causes of cancer were those associated with chemical exposure at the work place. These findings led to the concept that most human cancers were caused by environmental chemicals. For example, Hueper noted that the increasing rate of lung cancer coincided with the remarkable growth of industry and motorized transportation, as well as the use of industrial carcinogenic products and their release as effluents and exhausts into the occupational and general atmosphere. However, Wynder and Doll later showed that a major type of cancer, lung cancer, which was increasing sharply in men, was due to the chronic use of cigarettes and exposure to tobacco smoke.

One of the first indications that factors other than chemical interaction with specific intracellular sites could be responsible for tumor induction was the discovery of fibrosarcomas, observed around large bakelite discs implanted subcutaneously in rats. A similar observation was seen after wrapping pieces of cellophane film around the kidneys in rats. After several months, a large tumor developed around the cellophane film in some of the rats. The finding of "solid-state" carcinogenesis was confirmed later in the 1960s by implantation of various solids (including noble metals such as gold, silver, or platinum), which led to the development of sarcomas. The shape, and particularly the size of the implant, had a determining influence on sarcoma induction. In addition, iron-dextran was found to produce this type of tumor following repeated subcutaneous injections in rats and mice. Subsequent investigations revealed that some food colorings evoked local sarcomas in rats and mice after repeated subcutaneous administration. The principal mechanism believed to be involved in sarcoma induction was the continued activation of reparative processes in connective tissues by the physical, tissue-damaging properties. Further examples of chemical and solid-state carcinogenesis that lead to tumor development in rodents are provided by Grasso et al. (3) and Weissburger (4). Other recent evolutions in carcinogenicity concepts will be described further in this review.

History of Carcinogenicity Testing Legislation

One area of great contemporary concern was the entry of carcinogens into the human environment through food in the form of food additives and food contaminants, such as pesticides. Thus, hearings were held before the U.S. Congress relative to desirable modifications of the pure Food and Drug Laws that had been part of legislation since 1908. In 1958 and 1960, Congressman Delaney introduced the following two pieces of legislation, also known as the "Delaney Clause:"

1. Food Additives Amendment of 1958: Federal Food, Drug and Cosmetic Act. Section 409(c) (3) (A): "... no additive shall be deemed to be safe if it is found to induce cancer when ingested by man or animal, or if it is found, after tests which are appropriate for the evaluation of the safety of food additives, to induce cancer in man or animal..."
2. Color Additive Amendment of 1960; Federal Food, Drug and Cosmetic Act. Section 706 (b) (5) (B): "A color additive that will result in ingestion shall be deemed unsafe, and shall not be listed, ... if, after tests which are appropriate for the evaluation of the safety of additives for such use or after other relevant exposure of man or animal to such additive; it is found by the Secretary to induce cancer in man or animal."

This legislation consisted of the absolute prohibition of entry of "carcinogens" into the food chain, and established for the first time the view that any substance that could be considered carcinogenic was not to be added to foods or cosmetics. There was no room for quantification and calculation of risk. More detailed information on this clause can be found in the article by Weissburger (4).

From 1961 onward, the "Bioassay Program" of the National Cancer Institute (NCI) was developed. This program was based upon the need for a more systematic investigation of chemical carcinogenesis in animals (rats and mice). Each test also involved the use of appropriate negative and positive control carcinogens to verify that the models actually gave the appropriate results. Preliminary toxicology testing was performed using five rats or mice per group, and applying the dosage for 6 weeks, with a further holding period of 2 weeks without chemical administration to detect delayed toxicity. Animals that survived provided information on survival and weight gain (loss), the highest possible dose, and the maximum tolerated dose (MTD). The final NCI Chronic Bioassay consisted of a life-time (18–24 month) oral administration to rats and mice up to the MTD, with a second group at 50 or 30% of the selected MTD. The principle of the MTD was chosen since a number of bioassays in the literature

with the known human carcinogens gave a good incidence of cancer only at high dose levels. Between the 1960s and the 1980s, the NCI Bioassay Program tested many types of chemicals that humans were potentially exposed to, including chemicals used in cancer chemotherapy, hair dye formulations, and a series of agricultural chemicals, including pesticides, insecticides, and fungicides.

The NCI Chronic Bioassay became the basis for current guidelines on carcinogenicity testing. Protocols and reports for the large-scale bioassays were standardized in order to increase scientific acceptability.

A number of the chemicals administered were found to induce cancer in rats and mice. Some chemicals, particularly agricultural products, were negative in the rat models but induced liver tumors in mice. With either type of response, a chemical that induced cancer in any test was labeled a carcinogen, and by extrapolation was considered to be a human cancer risk without questioning its relevance or the underlying mechanism.

Towards the end of the 1980s, the NCI carcinogenesis branch merged with other agencies that shared similar efforts to form the National Toxicology Program (NTP). The NTP placed much more emphasis on mutagenicity and other short-term tests, teratology, behavioral effects, neurotoxicity, and various subchronic studies.

More information on the history of the Chronic Bioassay can be found in the reviews by Weissburger and Weissburger (5, 6).

Carcinogenicity Testing Guidelines

In the 1970s and 1980s, the US, European, and Japanese Registration Authorities established guidelines for carcinogenicity testing in animals for the various chemicals characterized by possible long-term intake by man. These chemicals included food and color additives, agrochemicals, industrial chemicals, solvents, human pharmaceuticals, and veterinary products. The guidelines were based upon the Chronic Bioassay of the NTP and gave indications for route and frequency of dosing, dose levels, group sizes, duration of the study, and observations during the study. A summary of the various guidelines is provided in Table 1. More detailed information on the various guidelines is provided by an overview of Inveresk Research International (7). Meanwhile newer versions of most of the guidelines have become effective.

CURRENT CARCINOGENICITY TESTING

A description of the standard approaches in carcinogenicity testing for the safety of chemicals is provided.

Standard Rodent Chronic Bioassay

Species and strains

According to the previously described guidelines, carcinogenicity studies have to be performed in two rodent species, usually the rat and the mouse. Ideally, the strains should have a low spontaneous incidence of cancer, but they should also be sensitive to induction of cancer by human carcinogens. Commonly used species are the Sprague Dawley, Fisher F344, or Wistar strains in rats, and the CD-1 or C57BL-based strains in mice. At least 50 animals are included per sex in each dose group. Today, at least three dose groups are used as well as at least one

Table 1 Establishment of guidelines for carcinogenicity testing in animals

Continent	Scope	Authority, year of approval
General	Chemicals	OECD, 1981
US	Food and color additives	FDA, 1982
	Agrochemicals	EPA–FIFRA, 1984
	Chemicals	EPA–TSCA, 1985
EC	Pharmaceuticals	EC directives, 1983
	Chemicals	EC directives, 1988
Japan	Agrochemicals	MAFF, 1985
	Veterinary products	MAFF, 1988
	Pharmaceuticals	MHW, 1989

EPA: Environmental Protection Agency; FDA: Food and Drug Administration; FIFRA: Federal Insecticide Fungicide and Rodenticide Act; MAFF: Ministry Agricultural Forestry an Fisheries; MHW: Ministry for Health and Welfare; OECD: Organisation for Economic Co-Operation and Development; TCSA: Toxic Substances Control Act.

negative control group, which results in minimally 200 males and 200 females for one study.

Doses and route of administration

The animals are exposed daily to the test compound from the age of 6 weeks onward. The administration procedure should simulate human exposure as closely as possible; oral intake is the most common route.

In the past, the test item was often mixed in the drinking water or the feed, either in a fixed concentration in the feed during the entire study or with regular adaptations to maintain a steady ratio of mg/kg of body weight intake during the entire life expectancy. Nowadays, oral gavage administration in the stomach is used, except for agrochemicals and food additives, where feed administration is still applicable. Oral gavage administration provides more certainty of test item intake, but also leads to another pattern of test item exposure in the body (peak concentrations after dosing). Dose-selection for the various dose groups has been based mainly upon a MTD, which is defined to elicit slight target organ toxicity but will not shorten the treated animals' survivability from any toxic effects other than the induction of neoplasms. For the most part, a body weight gain loss of 10% is considered acceptable as evidence of minimal toxicity. The medium dose may elicit minimal toxicity; however, the low dose should be free of any toxicity.

The MTD is mostly defined in a 3-month dose-range-finding study, allowing optimal dose setting for the pivotal chronic bioassay.

Duration

The studies are designed to last for at least 24 months and survival should be at least 25 animals per sex in the control and low dose groups, both in males and females. In the past, the studies were often extended beyond 24 months because survival (especially in the control and low dose group) was still above 25 animals/group/sex, and because of concern that the carcinogenic effect might become visible only at a later end point. Currently, most of the studies are not extended since geriatric pathology increases, which can complicate and obscure the assessment of carcinogenicity.

Experimental conditions

Experimental conditions are of utmost importance because they can influence the results of the study. Factors such as hygiene, temperature, relative humidity, number of air replacements, and light have to be maintained and monitored consistently during the study.

Today, carcinogenicity studies are performed under Specific Pathogen Free (SPF) conditions. This means that SPF animals are obtained from commercial breeders and are placed in SPF rooms after arrival in the experimental unit. Other factors taken into account are quarantine, health monitoring, and hygienic measures during handling, such as sterile gloves and mouth masks. Good Laboratory Practice (GLP) has also contributed to improved test conditions. GLP not only applies to the way animals are handled, but also to appropriate documentation and recording of all actions during a study. This leads to better traceability, reconstruction, and interpretation of study data and results. All the improvements in experimental conditions have led to increased survivability in the animals. In the past, various deaths occurred due to respiratory or other infectious diseases; these are almost totally excluded within the current improved health condition of the animals.

Parameters examined in the study

During the 24-month study, various study parameters are examined as shown in Table 2. The daily follow-up is of utmost importance in order to pick up unexpected findings. If problems arise, the study director's, veterinarian's, or pathologist's attention is drawn, and immediate and appropriate actions are requested. After necropsy during or at the end of the study, a mean list of 30 tissues is sampled and examined macroscopically. This may lead to a total number of 12,000 or more tissue samples for a single carcinogenicity study. All tissues are fixed and processed for further microscopic examination for neoplastic and nonneoplastic changes. These examinations are done by pathologists specialized in rodent pathology. The final aim is to detect the number of animals with tumors, but also multiplicity of tumors and whether the tumors caused death of the animal.

Histopathological evaluation

As indicated in Table 2, histopathological examination is performed on all animals to detect "nonneoplastic" and especially "neoplastic" changes induced by the test compound. Nonneoplastic changes may include inflammatory, degenerative, or other changes in various tissues, either caused by the test item or by geriatric pathology. Neoplastic changes, or tumors, can be divided by "benign" and "malignant" neoplasms. Benign neoplasms are well defined, often encapsulated, noninvasive, and well differentiated. They grow relative slowly, display relative few mitoses, and are not metastatic. Malignant tumors are less well defined and usually not well encapsulated. They are invasive and relatively undifferentiated; they grow rapidly, display abundant mitosis, and finally undergo metastasis).

Table 2 Parameters and frequency of observation or measurement in carcinogenicity studies

Frequency	Parameter	Examples of intended endpoint(s)
Daily	Mortality	Week of death or sacrifice Death or sacrifice status: Natural death or sacrificed in bad condition Terminal sacrifice
	Clinical observations	Drug-related signs of toxicity Bad condition, preceding mortality
Weekly	Body weight & weight gain	Drug-related body weight and weight gain or loss (10–15% body weight gain loss is accepted at MTD)
	Food consumption	Drug-related changes in food intake
After 6 or 12 months	Toxicokinetics	Exposure of parent compound and metabolites and relation to human exposure
After 6, 12, 18 months and terminally	Hematology	Drug-related haematological deviations
	Clinical biochemistry	Drug-related biochemical deviations
Terminally (also on all animals dying or sacrificed during the study except organ weights)	Gross pathology	Drug-related macroscopic findings
	Organ weights	Drug-related organ weight changes
	Histopathology	Nonneoplastic changes (including "pre-neoplastic" changes, such as hyperplasia) Neoplastic changes (tumors) Malignancy status (malignant or benign) Cause of death (tumors that caused death = probably fatal and fatal tumors; tumors that did not cause death = probably incidental and incidental tumors, and tumors in terminally sacrificed animals) Multiplicity of tumors (number of tumors/animal and/tissue)

Preceding conditions to neoplasia, such as "hyperplasia," (which refers to an increase in the number of cells) and "hypertrophy," (which refers to an increase in cell dimensions), can also be found.

Histopathological examination is a very intensive job; months may go by before all tissues are evaluated. In addition, the histopathologist will relate the histopathological findings with the other nonmorphological findings during the study, such as hematological or biochemical results, either for individual animals or for dosed groups. As such, diagnoses and mechanisms for the findings are established. For the most part, independent pathologists

perform peer reviews in order to have confirmation on the exact diagnosis or mechanism.

Historical control data

Historical control data can be used to interpret the changes seen in carcinogenicity data. These control data can apply to the various parameters studied, such as hematology, biochemistry, and the incidences of tumors. They may be used when differences are seen between the incidences of tumors in the dosed groups and spontaneous incidences in concurrent control animals, where coincidence is suspected, or for tumors with very sporadic incidences.

Spontaneous incidences in tumors are commonly seen in untreated rats and mice, and vary from strain to strain. Examples include pituitary and mammary tumors in rats, and liver and lung tumors in mice. The incidences of tumors can vary, and even today, there is no clear understanding of their etiology, except for ad-libitum feeding. On the other hand, caloric restriction could retard aging (associated with a reduction in the rate of cell replication), and reduce the incidence of degenerative diseases and tumor incidences (2).

Statistical analysis

Statistical analysis is performed on all parameters in the study. Its most fundamental objective is to determine whether administration of the test agent results in an increase in tumor incidence rates as compared to those in unexposed controls. Various statistical methods can be used. Tests for increased tumor occurrence rates between dosages may be based on "pair-wise comparisons," such as the Chi-square test for 2×2 tables, the Fisher's exact test, or the Cochan–Armitage test. These tests are most appropriate when survival rates do not differ appreciably in the various dose groups.

If the treatment results in reduced survival, early mortality in the high-dose groups may preclude the development of tumors and other statistical methods are required. Peto proposed a test for differences in tumor occurrence rates due to treatment, taking into account differences in survival and the times at which tumors were observed. This procedure requires information on the cause of death of each animal, and is based on a time-stratified contingency table analysis of the prevalence of incidental tumors that did not kill their host and a similar analysis of fatal tumors that resulted in death prior to the study. These two analyses are then combined to arrive at an overall test for increase in trend in tumor occurrence rates allowing for differential survival rates among the treatment groups. More information on study design and statistical analysis is provided by Feron et al. (8), Portier (9), Gart et al. (10), and Ciminera and Allen (11).

Cancer Risk Assessment

Once carcinogenicity testing has been performed, carcinogenicity risk assessment must be performed. Regulatory agencies have the responsibility to identify and assess compounds that are administered in food, provided as pharmaceuticals, or have the potential to be released in the environment at levels that warrant concern. Various topics have to be addressed when characterizing the carcinogenic risk. These include hazard identification (i.e., the likelihood to be a human carcinogen), dose-response, and extent of human exposure. Each of these assessments involve the use of many assumptions and estimations, the magnitude of which may be decreased by the incorporation of more information (e.g., mechanistic studies, pharmacokinetic data, and improved low dose extrapolation models). Velasquez et al. (12) have provided an overview of cancer risk assessment by the US Environmental Protection Agency (EPA) and biological issues in cancer risk assessment. In addition, the International Agency for Cancer Research (IARC) has evaluated and published carcinogenic risk to humans for hundreds of chemicals (13, 14).

In both systems, chemicals, including pharmaceuticals, are assigned to five groups: 1) carcinogenic to humans; 2) probably carcinogenic to humans; 3) possibly carcinogenic to humans; 4) not classifiable for human carcinogenicity; and 5) probably not carcinogenic to humans. Assignment to one of these groups is based on scientific judgement of data derived from studies in humans and animals as well as supporting data. Data are estimated as providing sufficient, limited, or inadequate evidence for carcinogenicity in humans and rodents.

For example, agents assigned as probably carcinogenic to humans have shown a positive association between exposure and human cancer, and have also shown sufficient evidence of carcinogenicity in animals. For the possible human carcinogens, only sufficient evidence for carcinogenicity in animals is established, but inadequate or no data in humans.

Evolution in Carcinogenicity Testing and Concepts Between 1960 and 2000

Mechanistic knowledge of carcinogenicity has increased enormously since the introduction of the Standard Chronic Bioassay. First, "genotoxic" carcinogens were distinguished from "nongenotoxic" carcinogens, which

Table 3 Main classification of chemical carcinogens

Category	Classification (+ example)	Features
Genotoxic	DNA-alkylating	Transspecies occurrence
	activation-independent (mustard gas)	At low dose levels
	activation-dependent (nitrosamines)	Short latency period
	Interfering with DNA (inorganic metals)	No threshold for extrapolation to humans
Epigenetic	Promotors (phenobarbital)	Mostly in one species, strain or sex
	Cytotoxic/mitogenic agents (*d*-limonene, saccharin)	At high dose levels (MTD)
	Hormone-modifiers (estrogen)	Long latency period
	Immunosuppressors (cyclophoshamide)	Threshold considerable for extrapolation to humans
	Peroxisome proliferators (clofibrate)	
	Receptor-mediated (dioxins)	
	Miscellaneous mechanisms (sodium nitrilotriacetic acid)	

led to a separate test battery for genotoxicity testing. Second, a "multistage" concept in carcinogenesis was discovered up to a molecular level. Third, "lifestyle factors" were found to play a major role in human cancer causes.

Genotoxic vs. nongenotoxic carcinogens

During the 1970s, Ames postulated that most human carcinogens were "genotoxic." Genotoxic compounds may react, either directly or indirectly (after metabolic activation) with DNA, which leads to alteration of the genetic material, and mostly to carcinogenicity in rodents and humans. On the other hand, many rodent carcinogens are "nongenotoxic" or "epigenetic," and act through a different mechanism.

The knowledge of these two basic mechanisms in carcinogenicity has led to the current classification of chemical carcinogens in "genotoxic carcinogens" and "nongenotoxic" or "epigenetic carcinogens." The second category comprises agents that exert primary cellular effects, which further result in carcinogenicity in rodents ("rodent carcinogens"). Genotoxic and nongenotoxic carcinogens, each with their own typical features, are further classified in main categories, as shown in Table 3. An example of an epigenetic rodent carcinogen is the artificial sweetener saccharin. In the past, when saccharin was shown to produce bladder tumors in rats, it was considered to be a likely (possible) human carcinogen. Today, it has been removed from the list of suspected human carcinogens since a large body of experimental data indicates that the rat bladder tumors arise from mechanisms that are not relevant to the human situation, which in turn supports the conclusion that saccharin is not related to bladder cancer in humans. Phenobarbital, a sedative, is an example of an agent that extensively induces drug-metabolizing enzymes in rodents. This results in liver tumors, but also in enhanced catabolism of thyroid hormones by the liver enzymes, which leads to thyroid stimulation by compensatory mechanisms and further to thyroid hypertrophy and hyperplasia, and eventually to thyroid tumors. Epidemiological studies, however, have indicated that it is of no concern for humans. More detailed explanations and examples of epigenetic carcinogens have been published by Purchase (15), Shaw and Jones (16), Van Cauteren et al. (17), and Williams and Whysner (18).

Due to the increase in knowledge of genotoxic and nongenotoxic mechanisms, test batteries were designed to evaluate "genotoxicity" in the early phases of drug development and prior to the start of chronic bioassays. These assays varied from test systems such as bacteria, yeast cells, and *Drosophila*-flies, to cell cultures of mouse lymphoma cells or human lymphocytes and in vivo systems, such as rodents for exposure of bone marrow. An overview of the genotoxicity toxicity assays that are currently performed for regulatory testing is shown in Table 4.

As indicated in Table 4, the potential to induce gene mutations or chromosome aberrations is investigated in a battery of genotoxicity studies. Additionally, DNA-damage and repair can be investigated separately. For the in vitro assays, some chemicals only become genotoxic after bioactivation to an electrophilic reactant. Therefore, in some in vitro assays, a liver enzymatic fraction is added in order to mimic the normal in vivo metabolic (de)activation route of compounds. The knowledge of the genotoxic versus the nongenotoxic carcinogens and of the mechanism of epigenetic carcinogenesis in rodents has gained significant importance in the extrapolation or relevance to humans. For example, humans have been

Table 4 Overview of genotoxicity assays

Genotoxicity assays	Genetic damage type
Assays according to current ICH-4 Standard battery:	
Ames reversion test in salmonella typhimurium or *Escherichia coli* (in vitro)	Gene mutations
Chromosomal aberration test in cell culture of human lymphocytes (in vitro) or	Chromosome aberrations
Mouse lymphoma test in mouse lymphoma cells (in vitro)	Chromosome aberrations and gene mutations
Micronucleus test in rodent bone marrow (in vivo)	Chromosome aberrations
Additional assays if cause for concern:	
Unscheduled DNA synthesis (UDS) test in rodent hepatocytes (in vitro/in vivo)	Primary DNA-damage
32P-postlabeling test	Primary DNA-damage
Test with transgenic animals	Gene mutations
Chromosome aberration test in human lymphocytes (in vitro) or in rodent bone marrow (in vivo)	Chromosome aberrations

exposed to many pesticides known to cause cancer in experimental animals by epigenetic mechanisms, but none has been definitely linked to cancer in humans. The absence of effects in humans is usually because exposure is below the threshold for the epigenetic effects. In addition, some cancer mechanisms may be specific to the rodent test animals and not relevant to humans at any possible exposure.

Multistage cancer concept

Armitage and Doll postulated the multistage cancer concept in the 1960s. According to this theory, tumors can arise from single cells that obtain proliferative capacity after several consecutive independent events at cellular level. Cancer of the colon is one of the best-understood examples of multistage carcinogenesis; it reveals at least six stages in its development. Recently, it also has become possible to identify the molecular events that underlie the development of these tumors. Some examples of somatic alterations in this model are gene mutations of a "ras" proto-oncogen, detected in approximately 50%, and loss of a "P 53" tumor suppressor gene in approximately 75% of the colorectal carcinomas. Similar alterations have also been detected in other human tumors, such as lung, breast, and brain tumors. More detailed information and examples of multistage carcinogenesis are provided by Fearon and Vogelstein (19), Couch (20), and Purchase (15). Based on the above theories, the main steps involved in carcinogenesis are: 1) "initiation" (first stimulus leading to alteration of the DNA in the cell nucleus); 2) "promotion" (further expression of the genetic change, leading to benign tumors); and 3) "progression" (further evolution of the tumor leading to malignancy and clinical manifestation).

"Initiation" is the first phase of carcinogenesis whereby cells are exposed to a carcinogenic agent. In this stage, an irrevocable step is taken in which daughter cells may later acquire relative autonomy with regard to cell division. "Promotion" is the next phase, in which initiated cells, not yet recognizable, are stimulated to divide and become clinically or pathologically detectable neoplasms. "Progression" is the phase in which the tumor increasingly damages the host and finally destroys it. Widespread invasion and metastasis, with destruction of the original normal tissue, are predominant in this phase.

Lifestyle factors

Finally, associated causes of major human cancers have been discovered. Most cancers are associated with lifestyle (specifically tobacco and excessive alcohol use), inappropriate nutritional traditions, and lack of exercise. These lifestyle components involve currently known genotoxic carcinogens, and importantly, nongenotoxic carcinogens. The effect of nongenotoxic carcinogens is highly dose-dependent and also reversible upon lowering the dose below a threshold. Thus, it is quite possible to lower human cancer risk as well as the risk of related chronic diseases, such as coronary heart disease, hypertension and stroke, and adult on-set diabetes, by proper lifestyle adjustments. Clearly, the Delaney Clause (requiring total absence of carcinogens in the food chain) plays no role in disease prevention (5). Misconceptions about the relationship between environmental pollution and human disease, particularly cancer, have also been highlighted by Ames and Gold (21). Smoking, dietary imbalances, chronic infections, and hormonal factors, all influenced by lifestyle, are considered major causes of cancer. Reduction in synthetic pesticides in the food chain does not effectively prevent diet related cancer, whereas high consumption of fruits and vegetables (containing antioxidants) does. On the other hand, humans do ingest many natural chemicals (in fruits and plants). Even though only a small proportion has been tested for carcinogenicity, half

of them are rodent carcinogens. Therefore, prevention of cancer will not only be based upon carcinogenicity testing and risk assessment of chemicals, but from knowledge obtained from biomedical research, education of the public, and lifestyle changes (21).

Need for Changes

Deficiencies of the standard chronic bioassay

If clear-cut evidence of genotoxic potential is obtained in the genotoxicity battery, especially via in vivo assays, then the potential for carcinogenicity in humans is highly suspected. In that case, drug development is discontinued. For compounds with negative or equivocal results in the genotoxicity battery and positive results in the Standard Chronic Bioassay, interpretation can be difficult.

The concept of the bioassay for detecting carcinogenic potential was developed at a time when relatively few agents were recognized as being carcinogens. However, during the past 20 years, many investigations have shown that it is possible to provoke a carcinogenic response in rodents by a wide diversity of experimental procedures, many of which are considered to have little or no relevance for human risk assessment. The outcome of the Standard Chronic Bioassay has been shown to be positive for about 50% of a random selection of chemicals in the US NTP program (22). These bioassays have frequently identified different target organs in rats and mice and even in different strains of one species. Further, the data from bioassays were often contradictory, with simultaneous increases and decreases of tumors in different tissues (23).

Drugs have to be administered at very high doses (MTD). The paradox is that the safer the chemical, the higher the MTD, and the more likely that biochemical distortions will lead to cellular injury or cell death, abnormal compensatory cell replication, toxic hyperplasia, and toxicity-induced cancer, which is not considered relevant to man (24). A comparative study between animal experiments and epidemiological studies on chemicals also showed that animal studies with test doses above the MTD resulted in carcinogenic effects in multiple organs that had no or limited predictive value for man (25).

The IARC has provided additional indications for the lack of human relevance by the chronic bioassay. IARC work groups have evaluated hundreds of pharmaceuticals for their carcinogenic properties. However, only 20 pharmaceuticals were conclusively carcinogenic to humans and 52 were "probably" or "possibly" carcinogenic (13, 14). The pharmaceuticals known to cause cancer in humans each possess at least one of the four properties: genotoxicity, immunosuppression, hormonal activity, or chronic irritation (cytotoxicity/mitogenic activity). These properties can be identified by genotoxicity studies in vivo and in well-designed toxicology studies of up to 6 months duration in rats (26). The absence of effects in humans was also considered to be due to an exposure in man below the threshold dose for the epigenetic effects or due to cancer mechanisms that were specific to the rodent test animal.

Therefore, the findings from nongenotoxic compounds can be shown to lack relevance for humans because either the animal exposure is excessive as compared to human exposure, or the response to a carcinogenic challenge is qualitatively different in rodents from that in humans (26). In case of a different mechanism, additional mechanistic studies often have to be performed to prove the lack of relevance to man.

International Conference on Harmonization (1991–1997)

Since 1991, the International Congress of Harmonization (ICH 1, 2, 3, and 4, respectively, taking place in 1991, 1993, 1995, and 1997) has met to promote harmonization of regulatory requirements between its regions (Japan, US, and Europe) on Safety, Quality, and Efficacy of human pharmaceuticals. Both regulatory authorities and industry associations were involved in these discussions.

Based upon the deficiencies of the standard chronic bioassay as well as on increased mechanistic knowledge, the following needed to be defined: 1) when carcinogenicity studies should be performed; 2) the use of new methods in carcinogenicity testing, and 3) guidance for dose selection (27). These three safety topics are described in more detail in Table 5.

As indicated in Table 5, carcinogenicity studies (see S1A) are only required for long-term human exposure of chemicals, but may also be recommended if there is cause for concern even with compounds lacking long-term exposure. ICH topic S1B takes into account the recent scientific developments, rather than harmonizing between nations. Several large databases of long-term rodent carcinogenicity studies conducted over the past 25 years indicate consistently that approximately 50% of chemicals yield a positive result. These pharmaceutical databases derived from pharmaceuticals only were based upon the shortcomings of the Standard Chronic Bioassay previously described. The pharmaceutical databases are summarized in Table 6.

The ICH addressed several main questions. These included: "Would the use of rats, but not mice, result in loss of information on carcinogenicity that would be relevant to human risk assessment?" and "Has a positive

Table 5 ICH topics on carcinogenicity testing

S1A. "Need for carcinogenicity studies"

Required for any pharmaceutical with expected "clinical use of at least 6 months"
Recommendation if there is "cause for concern:"

1. Previous demonstration of carcinogenic potential in a product class that is considered relevant to humans
2. Structure-activity relationship (SAR) suggesting carcinogenic risk
3. Evidence of preneoplastic lesions in repeated dose toxicity studies and
4. Long-term tissue retention of parent compound or metabolite(s) resulting in local tissue reactions or other pathological responses

S1B. "Testing for carcinogenicity of pharmaceuticals"

Flexibility and judgement should be exercised in the choice of approach.
Either 2 long-term carcinogenicity studies (one in the rat and one in the mouse) or one long-term study plus one other study with a shorter duration ("one plus approach").

S1C. "Dose selection in carcinogenicity studies"

Criteria for dose selection in carcinogenicity studies:

1. Toxicity-based endpoints (MTD)
2. Pharmacokinetic endpoints (25-fold AUC ratio: rodent-human)
3. Saturation of absorption (highest exposure reached)
4. Pharmacodynamic endpoints (e.g. sedation at high dose levels)
5. Maximum feasible dose (e.g. maximal solubility reached)
6. Other additional endpoints.

result in mice (but not in rats) correctly prevented a nongenotoxic drug from being marketed"? (32).

Results of the database surveys proposed that rats were more "sensitive" then mice and that tumorigenicity detected in mice only was never the sole reason for regulatory action. Furthermore, findings in rats only were twice as frequent as in mice only; all known human carcinogens were positive in rats (32). Therefore, it was proposed that normally one long-term study in one rodent species would suffice. The species should be the most appropriate and on practical convenience, the rat would be preferred. From the European point of view, one chronic bioassay in the rat would be sufficient. However from the US perspective, an additional short-term study was requested. The short-term models are further explained later in this article.

Finally, more criteria than the sole MTD were acceptable for dose selection (see Table 5, S1C). The availability of multiple acceptable criteria for dose selection provides greater flexibility in optimizing the design of carcinogenicity studies.

CARCINOGENICITY TESTING IN THE FUTURE

Alternative Carcinogenicity Models

According to ICH S1B, a "one plus approach" might be possible instead of the 2-year bioassay in rats and mice. In this approach, the species to be used in the one long-term study should first be selected, and secondly, the

Table 6 Overview of pharmaceutical databases

Continent	Scope	Reference
IARC (France)	International Agency for Cancer Research	13, 14
FDA (US)	Food and Drug Administration	28
PDR (US)	Physicians Desk Reference	29
JPMA (Japan)	Japanese Pharmaceutical Manufacturing Association	30
CPMP (EC)	Committee for Proprietary Medicinal Products	31
CMR (UK)	Center for Medicines Research	30

alternative model should be chosen, both based upon various selection criteria. Selection criteria for both the species and strain to be used in the long-term study and in the alternative model are summarized in Table 7. A description of the various alternative short-term models is given.

Transgenic mice

Transgenic mice may provide advantages in developing a more specific model and reducing the number of animals and the time required for bioassays (6–9 months daily dosing). Such models include transgenic mice that carry reporter genes that may serve as targets for genotoxic events or mice carrying specific oncogenes or inactivated tumor suppressor genes that are important factors that contribute to the multistage process of carcinogenesis.

Table 7 ICH S1B "testing for carcinogenicity of pharmaceuticals": Rationale

Select most appropriate species for the long-term study:

Based on comparative studies in two or more rodent species:

1. Pharmacology
2. Repeated dose toxicity studies
3. Metabolism
4. Toxicokinetics
5. Route of administration
6. In the absence of clear evidence favoring one species, the rat is recommended.

Select most appropriate alternative model:

Models:

1. Transgenic rodents (in vivo)
2. Neonatal rodents (in vivo)
3. Initiation–Promotion models (in vivo)
4. Syrian Hamster Embryonic cell (SHE) assay (in vitro)

Prior to inclusion of any new method, it is critical that the method be evaluated for:

1. Is new, additional information expected?
2. Will concerns be addressed?
3. Is there a comparable metabolism in man?
4. Is there a comparable exposure in man?
5. Is there literature or other evidence on the value and relevance of the model?

Mouse lines with defined genetic alterations that result in over-expression or inactivation of a gene intrinsic to carcinogenesis, but that are insufficient alone for neoplastic conversion, are promising models for identification and evaluation of rodent and/or human carcinogens. Likely models are:

1. Transgenic animals that over-express (proto-) oncogenes, such as:
   a. "TG.AC" mouse line (a "skin-painting model"), which expresses a mutation of the "v-ras" proto-oncogene (33).
   b. "Tg-rasH2" mouse line, which expresses a mutation and amplification of the human "c-Ha-ras" proto-oncogenes in different tissues (34).
2. Transgenic animals that lack certain genes ("knockout" animals), such as:
   a. "P53 +/−" mouse line, which expresses heterozygous inactivation of the P53 "tumor suppressor gene," which is critical to cell cycle control and DNA repair (33).
   b. "XPA−/−" mouse line, which expresses homozygous absence of "DNA-repair genes" (35).

Neonatal mice

Neonatal rodents have been studied since the 1960s. The test on newborn rodents (12 months of duration) has been extensively investigated in the United States and in Japan, and has been demonstrated to be highly sensitive to genotoxic carcinogens (36).

Meanwhile, further investigations have revealed that dosing on days 8 and 15 of age at the MTD and at half the MTD appears to be the current dosing regime, followed by a 12-month observation period.

The susceptibility of neonatal mice to tumorigenicity has been demonstrated and is explained by the fact that metabolic activity of a chemical in neonates is in the developing stage. Delay of excretion of a chemical via metabolic pathways results in prolongation of the presence of a chemical in the body. Such prolongation of the presence of a carcinogen would have a greater chance of changing normal cells into tumor cells.

Initiation—promotion models (in vivo)

The principle of this model is that in order to evaluate a substance as an "initiator," it will be administered in a single dose or over a period of several days or weeks. After several weeks of washout, a "promotor" (e.g., phenobarbital) is administered, and several months later, the number of pre-neoplastic or neoplastic changes are examined.

To evaluate whether an agent acts as a "promotor," the procedure is reversed. Following initiation with a known initiator (e.g., diethylnitrosamine), the substance under investigation is administered intermittently, at different dosages, over a number of months. In retrospect, this model might be valuable in establishing the mechanism underlying carcinogenicity (17).

Syrian hamster embryo (SHE) cell transformation assay (in vitro)

In this system, normal cells are isolated from 13-day-old embryos, and these cells are treated with carcinogenic agents capable of inducing genetic alterations that can produce changes in cellular and colony morphology that result in "morphologically transformed" SHE cells. These cells will senesce unless they undergo the additional genetic alterations necessary to acquire immortality and tumorigenicity. Several intermediate stages in this system, including morphological transformation, immortality, acquisition of tumorigenicity, and malignant progression, shape the alterations observed during in vivo neoplastic transformation and possibly explain why the SHE assay is able to identify rodent carcinogens (37). In the SHE assay, compounds are cultured over a period of 24 h–7 days, and colony formation is examined as the endpoint of clonal transformation.

Evaluation, Validation, and Implementation of the Alternative Models

Further description of alternative models and their evaluation and validation is given by Blain et al. (38). Validation exercises are taking place at the American National Institute of Environmental Health (NIEH), the International Life Science Institute/Health and Environmental Sciences Institute (ILSI/HESI) (39), the Japanese Central Institute for Experimental Animals/National Institute of Health Sciences (CIEA/NIHS), and the Dutch Public Institute for Safety and Environment (RIVM).

The NTP is also evaluating several lines of genetically-altered mice for possible use in identifying and assessing carcinogens. A number of comments and concerns were raised, offering some thoughts on future directions for this line of research as well as for the possible ways in which genetically altered mice might be integrated into a comprehensive testing strategy (40).

In the industry, the ICH S1B guidelines have been implemented, and have led to changes in carcinogenicity testing approaches, as well as changes in evaluation by the regulatory authorities. Guidelines increase flexibility, which in turn obligates industry and regulatory authorities to use more scientific, evidence-based decision-making. The changes are anticipated to significantly improve the relevance of the assessment of carcinogenic risk for humans, but might also lead to confusion and occasional disagreement on appropriate test strategies for specific drugs (41). Alternative test models might be used in various scenarios as follows: 1) as an alternative to a second 2-year rodent carcinogenicity study; 2) as a complementary confirmatory study for drugs with equivocal carcinogenicity findings in 2-year studies; 3) as a preliminary screen to set priorities for full carcinogenicity testing; 4) as an alternative to repeating a 2-year rodent carcinogenicity study; or 5) to assess the carcinogenic potential of genotoxic contaminants or degradation. Based upon available information, there is sufficient experience with some in vivo transgenic rodent carcinogenicity models to support their application as complementary second species studies in conjunction with a single 2-year rodent carcinogenicity study when appropriately justified (42). Therefore, a weight-of-evidence approach is justified.

Weight-of-Evidence Approach

A weight-of-evidence analysis on the level of suspicion for a carcinogenic risk should be performed based upon all available pre-clinical information that could raise concern for carcinogenicity. These concerns include carcinogenicity results, structure-activity-relationship (SAR), class evaluation, genotoxicity findings, repeated dose toxicity findings, interspecies comparison of pharmacodynamics, exposure and metabolism, and information on the intended clinical use, patient population, dose regimen, and pharmacokinetics and pharmacodynamics. SAR consists of finding the relationship between different (usually computable) characteristics of the molecules and the induced biological activity. The resulting model can be used for predicting (estimating) the biological activity, and in this case, carcinogenicity. Various commercial computerized systems are available for SAR, as described by Richard (43). However, the performance of SAR is still limited and affected by over sensitivity (43, 44).

Schwetz and Gaylor proposed a potential strategy for the weight-of-evidence approach that includes alternative carcinogenicity models (45). This strategy proposes alternate approaches, according to SAR, or findings in the toxicity and genotoxicity studies that would provide more mechanistic information.

Elements that should be included in the weight-of-evidence approach, including alternative models, are provided by Contrera and DeGeorge (42). These include evaluation of the results of carcinogenicity studies (including alternative models) and genotoxicity studies, as well as assessment factors in the consideration of the adequacy of the test models and risk-benefit considerations. It is apparent that different tests might be warranted for the various regulatory areas, such as pharmaceuticals, food additives, environmental, or industrial chemicals (42, 45).

Another option of performing a weight-of-evidence approach "before" starting the classical and/or alternative carcinogenicity bioassays is to submit a "Carcinogenicity Testing Strategy Paper" to the authorities (46). In this way, a dialogue between authorities and industry can be reached in order to effect an agreement on the approach and dose selection prior to initiation of pivotal studies.

In the United States, the Center for Drug Evaluation and Research (CDER) engages in dialogue with industry to reach agreements on "approach" and "dose selection" prior to initiation of pivotal studies (41). In addition, a procedure of scientific advice can be found in the European Medicines Evaluation Agency (EMEA)/Committee for Pharmaceutical and Medical Products (CPMP). This agency is open for questions regarding the requirement for a rationale. Companies are encouraged to inform the CPMP of the proposed carcinogenicity testing strategy (47).

Other Future Opportunities

Data from short to medium-term toxicity studies that precede carcinogenicity studies reveal that most of the nongenotoxic agents which induce tumors in rodents also produce other pathological changes in the tissues in which the tumors develop and at dose levels at which tumors are observed. These early changes range from altered hormone levels, impaired ion balance, and organ enlargement to specific and marked histopathological changes (48). These findings may be used for early detection of nongenotoxic carcinogens, and may also be extremely valuable for designing protocols for long-term bioassays. Furthermore, a thorough understanding of such early indicators will lead to the elucidation of specific mechanisms involved in carcinogenesis. Together with examination of possible thresholds for underlying toxic events, this confirms the basis for assessment of carcinogenic risk and for the regulation of human exposure.

Based upon the above rationale, a "tier approach in carcinogenicity testing and assessment" of pharmaceuticals can be followed, possibly with refinement, reduction, or replacement of test methodologies in carcinogenicity testing.

A first approach, according to the ICH S1A guideline scenario on the need for carcinogenicity testing, prescribes long-term carcinogenicity testing (in one or two species) for compounds with continuous or intermittent exposure to humans and compounds with cause for concern. If there is no long-term exposure to the compound, and if there is no cause for concern, no further action is recommended, whereas short- or long-term studies may be warranted for suspicious findings (46).

A second approach postulates that much of the information necessary to assess the carcinogenic potential of a new drug without a bioassay is usually available by the end of the first clinical studies in patients. (Suspicious findings from in vivo genotoxicity studies and 3–6 month toxicology studies aimed at assessing risk factors associated with carcinogenicity in humans include: genotoxicity, immune suppression, hormonal activity, and chronic irritation/mitogenic activity.) Evaluation of this package will, therefore, identify the presence or absence of the known causes of cancer from pharmaceuticals in humans, under conditions relevant to the use of the drug in question. If cause for concern remains at this stage, useful information on long-term adverse effects that might represent a carcinogenic hazard to humans may be obtained (e.g., from a 12-month study, usually in rats, conducted at clinically relevant dose levels) (49).

Finally, a third approach has been proposed with five stages that focus on the chemical structure, DNA-reactivity, epigenetic effects, limited bioassays, and finally, the application of "accelerated bioassays." These accelerated bioassays require 40 weeks and apply to the use of sensitive markers for induction of neoplasia in comparison to positive control compounds for important organs in human carcinogenesis. It enables data acquisition of the entire carcinogenesis process directed toward developing mechanistic information. This system would have the potential to replace the chronic bioassay in rodents in some circumstances and could serve a analternative to a chronic bioassay in a second species (50).

CONCLUSION

In the 20th century, the concept of carcinogenesis and carcinogenicity testing has evolved enormously, although the standard Chronic Bioassay still contains many of deficiencies. New carcinogenicity testing strategies, however, are to be expected. Also, validation results

with regards to the alternative carcinogenicity models will become available and lead into new insights in the most appropriate short-term carcinogenicity studies.

A weight-of-evidence or tier approach to the level of suspicion of carcinogenic risk will be the main objective, and will include SAR and findings from sub-chronic to chronic toxicity testing (genotoxicity, reproductive toxicity, and organ toxicity). In addition, communication between industry and regulatory authorities will be formulated early in order to allow the most optimal scientific approach.

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